Supporting Information

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SI Text

Synthetic siRNA Molecules. The target sequences of the different siRNA molecules used in this study are the following: Mcm2 (5′ GGAGCU CAUUGG AGAUGG CAUGGA A), Mcm3 (5′ GCAUUG UCACUA AAUGUU CUCUAG U), Mcm4 (5′ CCAUUG AGUCUC AGUGGA AUCCUA A), Mcm5 (5′ CCCAGC UUCUGA AGUUUG UGGAGA A), Mcm6 (5′ GCGAAU CCUCGG CACUAA GCAAAU A) and Mcm7 (5′ GGUAGA AGGAGA GAACAC AAGGAU U).

Estimation of Fork Progression Rate and Origin Density by DNA Combing. To estimate fork progression rate, cells were pulsed with 100 μM BrdU for 30 min. To measure origin activity, cells were pulsed sequentially with 50 μM IdU for 15 min and 100 μM CldU for 25 min. In all cases, 1 mM thymidine was added to the medium for 1 h before cell collection. When indicated, 10 nM UCN-01 was added to the medium for 5h to inhibit Chk1 activity, before the pulse with nucleotide analogues. Genomic DNA was purified in agarose plugs and DNA was "combed" onto coverslips as described (1). Coverslips were incubated in 1 M NaOH for 25 min, neutralized in PBS (pH 7.5), and processed for immunofluorescence. BrdU or CldU were detected with rat anti-BrdU monoclonal antibody (SeraLab). IdU was detected with mouse anti-BrdU (clone BD44; BD Biosciences PharMingen). ssDNA was visualized with an anti-DNA antibody (Chemicon). Images were collected and analyzed with MetaMorph 7 software (Molecular Devices).

Fork progression rates were calculated as the average length of BrdU tracks divided by the time of the pulse. At least 100 BrdU tracks were counted. Technically, each BrdU track could reflect either a moving fork or a bidirectional origin of replication. However, using the dual IdU/CldU labeling protocol (see below), we estimated that the "bicolor" signals corresponding to moving forks were 8-fold more abundant than the "tricolor" signals corresponding to origins of replication. Therefore the length of BrdU stretches is a good representation of fork progression speed.

Relative origin density was estimated after the dual pulse with IdU and CldU as the number of active origins visualized by DNA combing relative to the total length of DNA analyzed for each cell population. If IdU and CldU are detected with red and green fluorescence marks, respectively, the signature of an origin that fires during the time of the double pulse is a tri-color label (green-red-green). Origins that had fired shortly before the pulse can be detected by the pattern (green-red-DNA-red-green). Bi-color signatures (green-red or red-green) indicate replication forks moving in one or other direction. "Termination structures" (red-green-red) reflect two head-to head colliding forks. Interspersed label (green-red-green-red-green) may indicate unchecked activation of neighbor replication origins (see Fig S4). Tri-color signals were spotted manually by two independent scientists, and the results averaged. For the quantification of total DNA, immunofluorescence images from labeled DNA fibers were processed with Metamorph 7 to eliminate background and measure the length of individual DNA fiber in the field. A cut-off size of 50 Kb was implemented to avoid small or broken DNA fibers.

1. Michalet X, *et al.* (1997) Dynamic molecular combing: Stretching the whole human genome for high-resolution studies. *Science* 277:1518–1523.

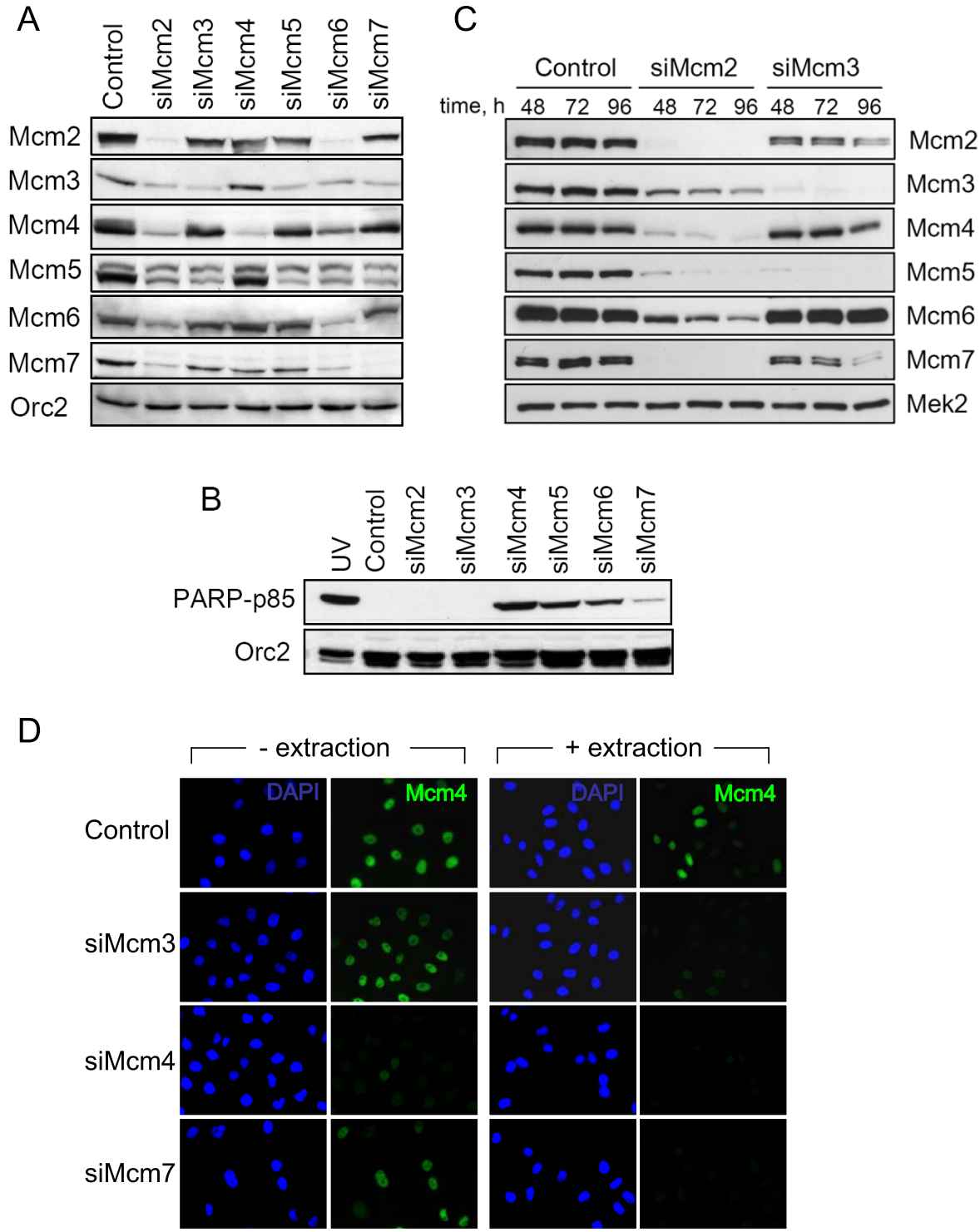
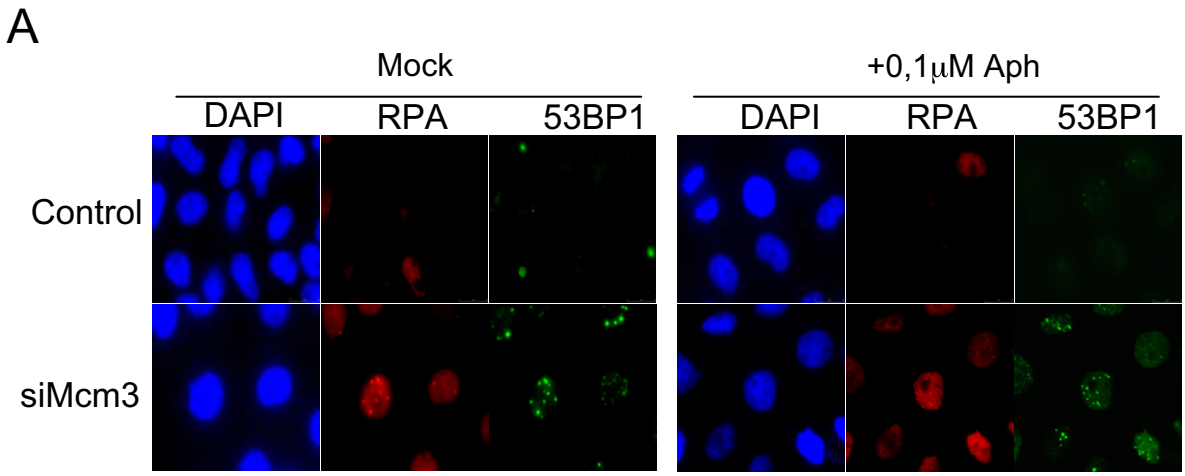
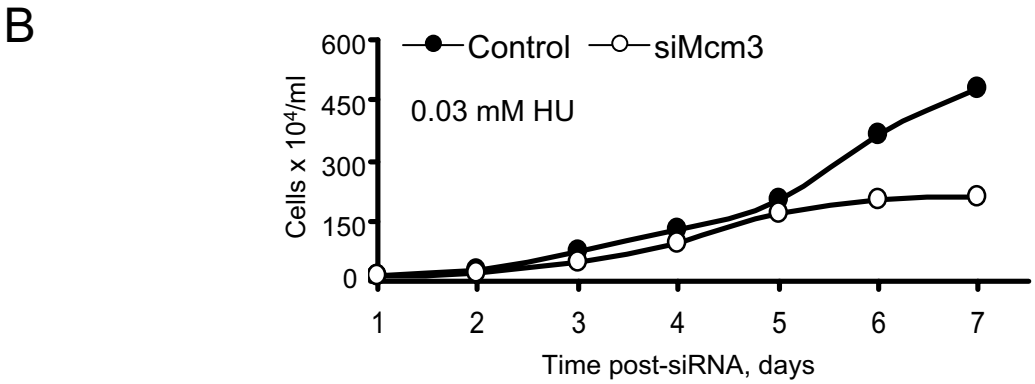


Fig. S1. Reduction in MCM protein levels and activation of apoptosis after Mcm2–7 siRNA. (*A*) Levels of individual MCM proteins in HeLa total cell extracts prepared 24 h after the transfection of the indicated siRNAs, determined by immunoblots with specific antibodies. Orc2 is shown as loading control. (*B*) Activation of an apoptotic response 72 h after trasnsfection with the Mcm4–7 siRNAs. The caspase-cleaved fragment p85 of Poly(ADP-ribose) polymerase (PARP-p85) was detected by immunoblot. As a positive control of apoptosis activation, HeLa cells were exposed to UV radiation (1,000 J/m2) in a UV Stratalinker (Stratagene) and incubated for 5 h at 37°C before harvesting and extract preparation. Orc2 is shown as loading control. (*C*) HeLa cells treated with the indicated siRNAs were subjected to a biochemical fractionation protocol to separate soluble from chromatin-enriched fractions (*Material and Methods*). The levels of soluble Mcm2–7 proteins are shown. Cytosolic kinase Mek2 serves as a loading control. (*D*) Indirect immunostaining of total Mcm4 protein or chromatin-bound Mcm4 after detergent preextraction of the soluble nuclear proteins before cell fixation (*Material and Methods*) in control cells or cells treated with the indicated siRNAs. DNA was stained with DAPI (blue).





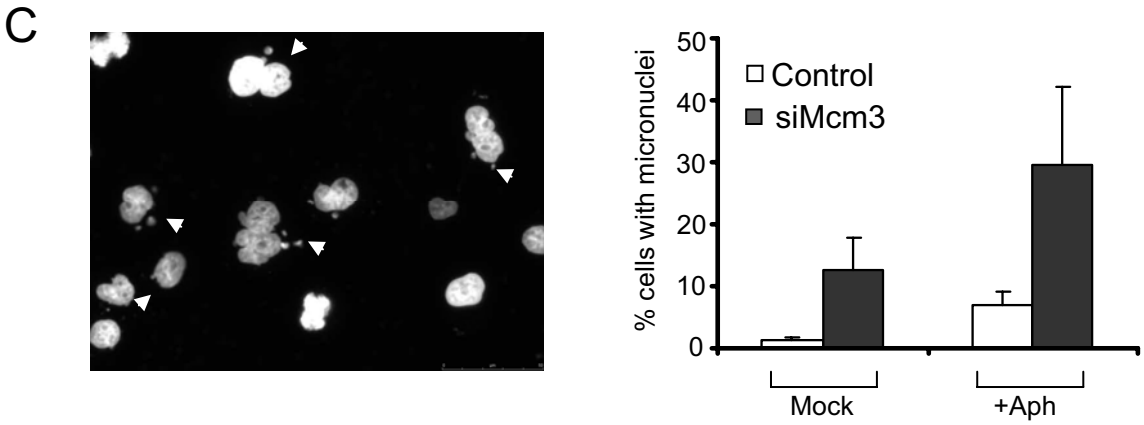


Fig. S2. DNA damage, hypersensitivity to hydroxyurea, and formation of micronuclei in cells treated with Mcm3 siRNA. (*A*) Immunostaining of RPA (red) and 53BP1 (green) in control cells or cells transfected with Mcm3 siRNA, grown in the absence (mock) or in the presence of 0.1 μM aphidicolin. DNA was stained with DAPI (blue). (*B*) Proliferation curves of control (closed circles) or Mcm3 siRNA-treated cells (open circles), in medium supplemented with 0.03 mM hydroxyurea. (*C Left*) DNA staining with DAPI (white) of a representative field of cells treated with Mcm3 siRNA and 0.1 μM aphidicolin. Arrows indicate micronuclei. (*Right*) The bar graph indicates the percentage of cells that presented micronuclei ($n > 200$ in each case), grown in regular medium (mock) or in the presence of 0.1 μM aphidicolin.

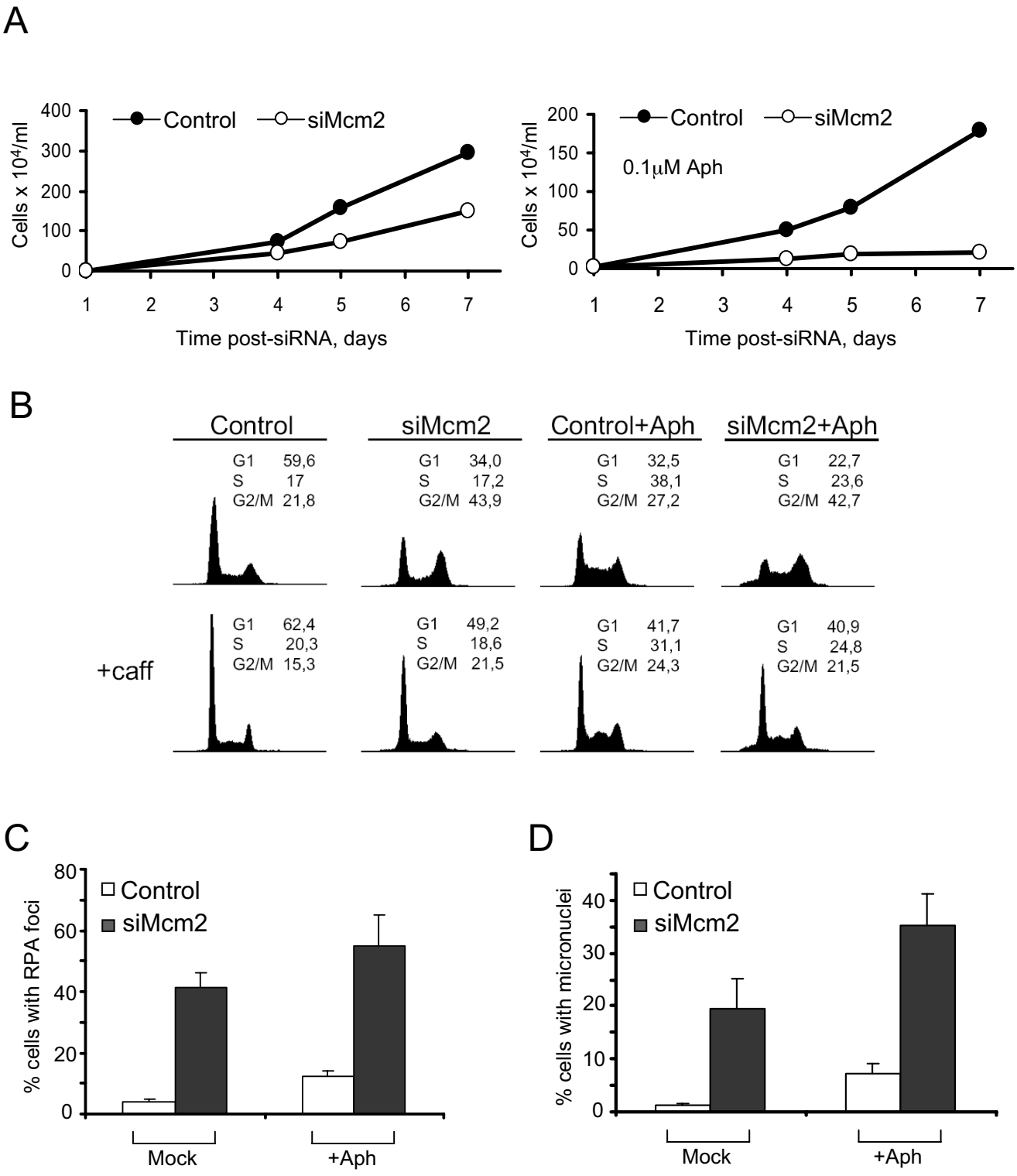


Fig. S3. Hypersensitivity to replication stress and activation of the DDR in cells treated with Mcm2 siRNA. (*A*) Proliferation curves of control (filled circles) or Mcm2 siRNA-treated (open circles) cells, grown in regular medium (*Left*) or in medium supplemented with 0.1 μM aphidicolin (Right). (*B*) DNA content of control cells or cells treated with Mcm2 siRNA, analyzed 120 h after transfection. +Aph indicates the presence of 0.1 μM aphidicolin in the medium. +Caff indicates treatment with 5 mM caffeine for 5 h before cell collection and analysis. (*C*) Percentage of cells that presented RPA foci after the treatment with Mcm2 siRNA, grown in the absence or in the presence of 0.1 μM aphidicolin ($n > 200$ in each case). (*D*) Percentage of control or Mcm2 siRNA-treated cells that presented micronuclei when grown in regular medium (mock) or medium supplemented with 0.1 μM aphidicolin ($n > 200$ in each case).

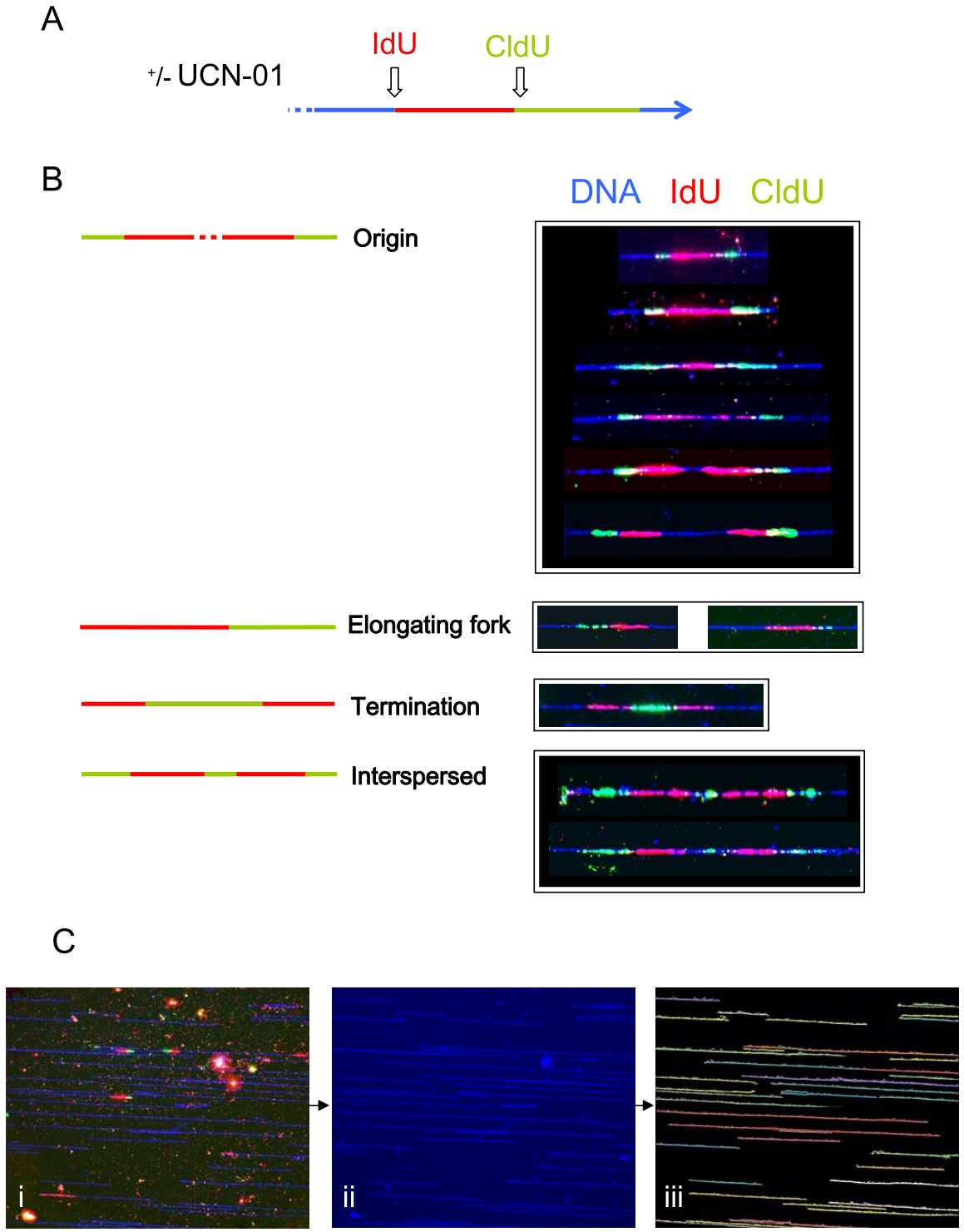


Fig. S4. Analysis of DNA replication origins by DNA molecular combing. (*A*) Schematic of the two pulses with nucleotide analogues to label newly synthesized DNA. Asynchronously growing HeLa cells were first treated with IdU for 15 min, followed by a second pulse with CldU for 25 min (see *Materials and Methods*). (*B*) Representative examples of the four main classes of replication structures detected after DNA combing and IdU (red)/ CldU (green), DNA (blue) immunodetection: replication origins, elongating forks, terminations, and interspersed regions. Origin density was estimated in each experimental condition by dividing the number of bona fide origins by the total length of DNA examined. (*C*) Example of quantification of total DNA. Raw immunofluorescence images from labeled DNA fibers (*i*) were processed with the Metamorph 7 software to eliminate all color information not corresponding to DNA (*ii*). The software detects each individual DNA fiber in the field and measures its length (*iii*).